



## Leaf Phytognostic Characters of Six Species of *Hypericum* L. (Hypericaceae)

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**Abstract:** Most of the studies on *Hypericum* genus deals with the phytochemical and pharmacological properties and the morphoanatomical data are scarce, although sometimes some species are poorly identified. In order to obtain differentiating phytognostic characters, the micromorphological, anatomical and histochemical study of mature leaves of six *Hypericum* species, *H. androsaemum*, *H. calycinum*, *H. canariense*, *H. grandifolium*, *H. x inodorum* and *H. patulum*, was carried out using different techniques of microscopy. Qualitative characters of both epidermal surfaces were evaluated: cell shape, cell wall, epicuticular deposits and stomata type. Only the last three characters showed some kind of differences. Five qualitative characters, stomata and gland indexes, total mesophyll thickness, palisade parenchyma and spongy parenchyma thickness, were subjected to analysis of variance on one factor ANOVA. Differences were found in the stomata and gland indexes as well as in the parenchyma cells arrangement and thickness. Histochemical tests were performed to localize and identify chemical groups of metabolites and the differences found were semi-quantitative. We conclude that the most important leaf phytognostic characters are: the epidermal cells shape, the type and distribution of stomata, the glands distribution and the mesophyll features.

**Keywords:** *Hypericum*, micromorphology, anatomy, leaf epidermal surface, mesophyll.

### I. INTRODUCTION

The genus *Hypericum* L., Hypericaceae Juss. [1] is originated from Eurasia and is widely distributed in tropical and subtropical regions. It includes approximately 480 species, classified into 36 Sections [2]. Most *Hypericum* species show one or more different types of secretory structures in their vegetative and reproductive organs: dark glands, translucent glands and secretory channels [3-5]. It is at the level of these structures that the synthesis and accumulation of biologically active substances happens, for instance naphthodianthrone, flavonoids and phloroglucinol derivatives [6], to which important medicinal effects are attributed, such as antiretroviral and antidepressive action [7-8]. *H.*

*perforatum* L. (St. John's wort) is the most studied species and the only one recognized by occidental Pharmacopoeias, but there are some other species of *Hypericum* used as traditional medicinal plants to treat a variety of ailments [8-12]. The phytochemistry diversity and the bioactivity have been investigated in many species, but their morphological characteristics have been forgotten. However, due to geographical differences, apomictic reproduction modes [10] and the existence of several hybrids [13], the identification of some species might be difficult [14-17]. It is accepted that the micromorphological approach is more accurate, less environmental influenced, complementary to the macrocharacters and advisable in situations where the species limits is obscure [18].

*H. androsaemum* L. is one of the eleven *Hypericum* species found in continental Portugal [19-20]. In traditional medicine it is often used as a hepatic protective [21-22] instead of *H. perforatum*. *H. androsaemum* is subject to misidentification and adulterations by other species, some of them introduced species but already naturalized, such as *H. x inodorum* Mill. and *H. patulum* Thunb. [22]. Reports on *H. androsaemum* phytochemistry and bioactivities are known [21; 23-24] and also on chemotaxonomy involving this and other Portuguese species [25] but little is known about their phytognostic characters. Maggi et al., [17] and Perrone et al. [26] documented the morphology and histochemistry of Italian populations of *H. androsaemum*. These authors mentioned that there are some differences in size and shape of leaves and flowers, type and distribution of the secretory structures on the herb's parts. The histochemical study allowed the localization of hypericin, flavonoids and essential oils. Hyperforin (that reaches the highest concentration in *H. perforatum*) is absent in *H. androsaemum* [17]. In this work we highlight six species: *H. androsaemum*, *H. calycinum* L., *H. canariense* L., *H. grandifolium* Choisy, *H. x inodorum* Mill. and *H. patulum* Thunb. They all are spontaneous or introduced shrubs, with morphologically similar leaves and sometimes are incorrectly identified and confused with each other. Their leaves do not have dark glands, they only show translucent

glands. Considering that herbals usually are fragmented or even powdered, phytognostic characters can only be obtained by micromorphological examinations. These characters enable a comparative study and supply a valid criterion for species morphological differentiation, as well as the accurate and reproducible herbals quality control. That is the reason why microscopy data are part of the Pharmacopoeias plant monographs.

The present work reports the microscopic evaluation of mature leaves of the mentioned species, comparing their morphoanatomic and histochemical traits. To this end, imaging techniques such as scanning electron microscopy, conventional light and fluorescence microscopy, as well as staining techniques, were used.

## II. MATERIALS AND METHODS

### Plant material

*H. androsaemum*, *H. calycinum*, *H. canariense*, *H. grandifolium*, *H. x inodorum* and *H. patulum*, were kept in culture in Parque Botânico of Tapada da Ajuda, Instituto Superior de Agronomia, University of Lisbon, Portugal. Shoots were collected before flowering and voucher specimens were deposited at the LISI Herbarium of ISA (Table 1). Each species was represented in the study by fifteen different fully expanded leaves, each one from a different shoot.

**Table 1.** *Hypericum* species identification.

Taxa	Section	Voucher (LISI)
<i>H. androsaemum</i> L.	Androsaemum (Duh.) Godr.	1864/2009
<i>H. calycinum</i> L.	Ascyreia Choisy Eremante (Spach) Blume	1767/2009
<i>H. canariensis</i> L.	Webbia (Spach) R. Keller	771/2008
<i>H. grandifolium</i> Choisy	Androsaemum (Duh.) Godr.	1766/2009
<i>H. x inodorum</i> Mill.	Androsaemum (Duh.) Godr.	773/2008
<i>H. patulum</i> Thunb.	Ascyreia Choisy	363/2007



### Analysis of plant material

A part of the plant material was cleared with chloral hydrate solution [27]. The observation of epidermal cells, stomata and gland distribution was estimated in the mid-lamina of both leaf epidermal surfaces. Stomata type followed Stace classification [18] and stomata index was found according to the European Pharmacopoeia 8.0 [27]. Histochemical tests and appropriate controls were performed on hand-sectioned fresh material: Sudan III for total lipids [28]; Nile Blue for neutral and acidic lipids [29]; Cu acetate / Rubeanic acid for fatty acids [30]; Nadi reagent for essential oils and resins [31]; 2,4-Dinitrophenylhydrazin for terpenes with a carbonyl group [30]; Anthimonium chloride for steroids [32]; Ferric chloride and Potassium dichromate for phenolic compounds [28]; Vanillin/HCL for tannins [33]; Tannic acid./ferric chloride for mucilages and pectins [34]; PAS for polysaccharides [35]; Dittmar reagent for alkaloids [36]. Light microscopy observations were made with a Nikon Labophot 2 LM and images were obtained with a Nikon325 FX-35W camera. The histochemical characterization was complemented with auto fluorescence and induced fluorescence observations with aluminum trichloride as fluorochrome for poliphenols detection on an epi-fluorescence microscope Olympus BX60 with an image acquisition system using an Olympus DP 50.

Other part of the plant material was fixed for scanning electronic microscopy (SEM), according to the usual methods [37]. SEM observations were carried out at 15 kV on a JSM-5220 LV, equipped with image acquisition. For anatomical studies the fixed plant material was processed with the paraffin micro technique and sections were stained with acidic phloroglucin [38]. All

measurements and counts related with these micromorphological characteristics were done on random fields, always at comparable leaf situations and magnifications.

### Data analysis

Data analysis was performed statistically using the SAS® program (SAS Institute, Cary, NC, USA). Data were subjected to analysis of variance on one factor ANOVA, with a significance level of 5%. Means of morphological characters were separated using Fisher's Protected LSD test at the 5% level of significance.

## III. RESULTS AND DISCUSSION

The comparative study of the epidermal surfaces of the examined species provided some elements for their recognition and selection (Table 2). Adaxial and abaxial epidermal cells have polygonal shape, with anticlinal cell walls almost straight (Table 2). Only *H. androsaemum* (Fig. 1A) and *H. calycinum* (Fig. 1B) showed slightly sinuous cell walls on both surfaces. Esau [39] assigns to the sinuous cell wall an important mechanical function, increasing the rigidity of the parietal cell to prevent collapse in case of water deficits and its occurrence might be considered as a result of difficult environmental conditions such as disability in light and humidity. In all the samples the epicuticular waxes were also noticed in the leaf epidermis as large platelets, randomly oriented, showing a similar distribution in both faces (Table 2), although denser on the adaxial side of *H. canariense* (Fig. 1C), *H. androsaemum* and *H. calycinum*. Perrone et al., [26] mentioned that the cuticular ornamentations in *H. androsaemum* are scarce and barely detected, which is not in agreement with the one found in our samples.

**Table 2.** Characters of the epidermal surface of the species of *Hypericum* under study. (HYPAN, *H. androsaemum*; HYPCA, *H. calycinum*; HYPCN, *H. canariense*; HYPGF, *H. grandifolium*; HYPIN, *H. x inodorum*; HYPPT, *H. patulum*; Ad., adaxial; Ab., abaxial; (+) slightly; (++) medium; (+++) high; nd, not defined).

<b>Epidermal surface characters</b>	<b>HYPAN</b>	<b>HYPCA</b>	<b>HYPCN</b>	<b>HYPGF</b>	<b>HYPIN</b>	<b>HYPPT</b>
<b>Ad. cell shape</b>	poligonal	poligonal	poligonal	poligonal	poligonal	poligonal
<b>Ad. cell wall</b>	sinuous (+)	sinuous (++)	straight	straight	Straight	sinuous (+)
<b>Epicuticular</b>	random	random	random	random	random	random
<b>Ad. Deposit (density)</b>	orientated platelets (+++)	orientated platelets (++)	orientated platelets (+++)	orientated platelets (+)	orientated platelets (+)	orientated platelets (+)
<b>Ab. cell shape</b>	poligonal	poligonal	poligonal	poligonal	poligonal	papillate
<b>Ab. cell wall</b>	sinuous (++)	sinuous (++)	sinuous (++)	sinuous (+)	sinuous (+)	nd
<b>Epicuticular</b>	random	random	random	random	random	random
<b>Ab. Deposit (density)</b>	orientated platelets (+++)	orientated platelets (++)	orientated platelets (++)	orientated platelets (++)	orientated platelets (+)	orientated platelets (+)
<b>Ab. stomata type</b>	anomocytic anisocytic	anisocytic	anomocytic anisocytic	anomocytic brachyparacytic	anomocytic brachyparacytic	nd

All species show stomata in the abaxial epidermis (Table 2): anomocytic and anisocytic in *H. androsaemum* (Fig. 1A) and *H. canariense*; anisocytic in *H. calycinum* (Fig. 1B); anomocytic and brachyparacytic in *H. grandifolium* (Fig. 1D) and in *H. x inodorum*; not defined in *H. patulum* because this taxon showed convex epidermal cells forming small papilla which turns difficult the stomata observation and classification (Fig. 1E). Epidermis papillose was also reported in certain species of *Hypericum* by Metcalfe e Chalk [40]. These authors also mentioned that in this genus

the stomata are usually confined to the lower surface, commonly surrounded by three or more cells and there are cases in which several types have been recorded in a single leaf, just like what we observed, with the exception of *H. calycinum*. Perrone et al. [26] also refer that *H. androsaemum* is a hypostomatic species, displaying only anisocytic stomata, which is in disagreement with our observations. Some researchers consider that stomata index is useful in comparing leaves of different sizes and they agree that the leaf mid-lamina of the abaxial



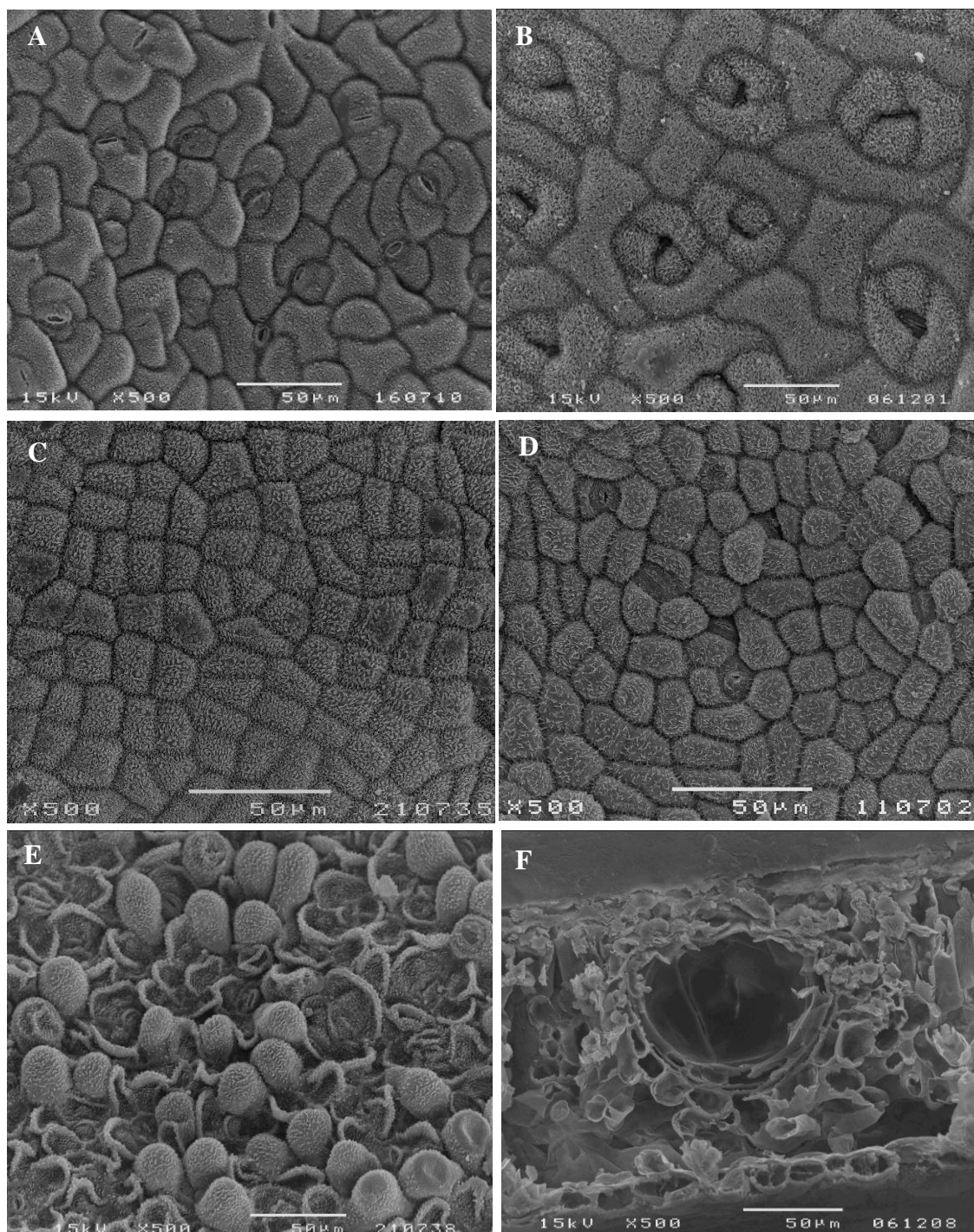


Figure 1. SEM micrographs of foliar epidermal surfaces: A - *H. androsaemum* abaxial epidermal cells, anomocytic and anisocytic stomata; B - *H. calycinum* abaxial epidermal cells and anisocytic stomata; C - *H. canariense* adaxial epidermal cells; D - *H. grandifolium* abaxial epidermal cells, anomocytic and brachyparacytic stomata (arrow); E - *H. patulum* abaxial epidermal papillate cells; F - *H. calycinum* leaf cross section with a cavity type internal secretory structure.

surface is the least variable and therefore most suitable for such measurements [Rowson in 41; 42; 18; 43]. Concerning the stomata index values,

three groups were found, with *H. patulum* being isolated with significant highest values, *H.*



androsaemum significant lower values and the other four taxa with values in between (Fig. 2A).

The genus *Hypericum* can show different types of glandular structures in the aerial parts, translucent glands, secretory canals and black nodules, pointing to an intense secretory activity, but some species only have translucent glands in their leaves [40; 44; 3-4; 17]. The latter situation is found in all the studied taxa, where the glands are randomly distributed, usually small and more or less rounded in shape.

*H. calycinum* has the bigger translucent glands but their number was very low as the gland index was the lowest (1.0%) among the species under study. On the opposite is *H. canariense* (Fig 2B) with the highest gland index (39.5%). Glandular structures are genetic features of certain species, thus are formed without the influence of external factors but their density may or may not depend on external factors, such as injuries, pathogens or physiological stresses [45]. In transverse sections, the secretory glands were seen as cavities delimited by a flattened layer of secretory cells and by another external layer of rounded and thicker walled cells (Fig. 1F). They appear in the vicinity of the veins and sometimes in association with them. This suggests a certain connection between those two types of tissues [39]. As noticed on Table 3, there are no significant qualitative differences in the specimens'

compounds. The secretion accumulated inside translucent glands stains positively for some lipophilic compounds: total lipids (Fig. 3A), acid lipids (Fig. 3B), fatty acids, essential oils (Fig. 3C) and for alkaloids (Fig. 3D). According to Costa [46], the positive results obtained for alkaloids should be interpreted with caution, since the reagents used can react with other different nitrogenous compounds such as proteins and amines. Steroids stained slightly positive just in *H. patulum* glands (Table 3). These results are somehow in accordance with some other studies on *Hypericum* that mention the presence of lipids and essential oils on translucent glands [7; 44; 4; 17; 47]. Phenolics were not detected inside of any species translucent glands at all and a similar situation was seen with the tannins, the mucilages and the polysaccharide histochemical tests (Table 3). In some situations their presence was noticed on cell walls of the cell layers limiting the glandular structures and on all the parenchyma cells (Fig. 3E). Under UV light sometimes slightly blue auto fluorescence was also noticed on the cells surrounding the translucent glands (Fig. 3F), pointing to the presence of some phenolic compounds. Some flavonoids are known to accumulate on leaf surfaces and play a role in the protection against UV radiation [48] which might explain the fact that they were more noticed on superficial glands.

**Table 3.** Histochemical tests on the content of the translucent glands of the *Hypericum* species studied. (HYPAN, *H. androsaemum*; HYPCA, *H. calycinum*; HYPCN, *H. canariense*; HYPGF, *H. grandifolium*; HYPIN, *H. x inodorum*; HYPPT, *H. patulum*; - negative; + slightly positive; ++ positive; +++ strongly positive; nd. not defined).

Histochemical Tests	HYPAN	HYPCA	HYPCN	HYPGF	HYPIN	HYPPT
Sudan III (total lipids)	+++	+++	+++	+++	+++	+++
Nile Blue (acidic lipids)	++	+++	+++	+++	+++	+++
Cu Acetat / Rubeanic ac. (fatty ac.)	+++	+	+	+	+	nd.



Nadi Reagent (essential oils and resins)		++	++	+	+	++	++
2,4- Dinitro-phenilhidrazin (terpenes)		-	-	-	-	-	-
Anthimonium chloride (steroids)		-	-	-	-	-	+
Ferric chloride (phenolics)		-	-	-	-	-	-
Potassium dichromate - (phenolics)	-	-	-	-	-	-	-
Vanillin /HCL (tannins)	-	-	-	-	-	-	-
Tannic ac./ferric chloride (mucilages/pectins)	+	+	+	+	+	+	+
PAS (polysaccharides)	-	-	-	-	-	-	-
Dittmar Reagent (alkaloids)	+	++	++	++	++	++	++

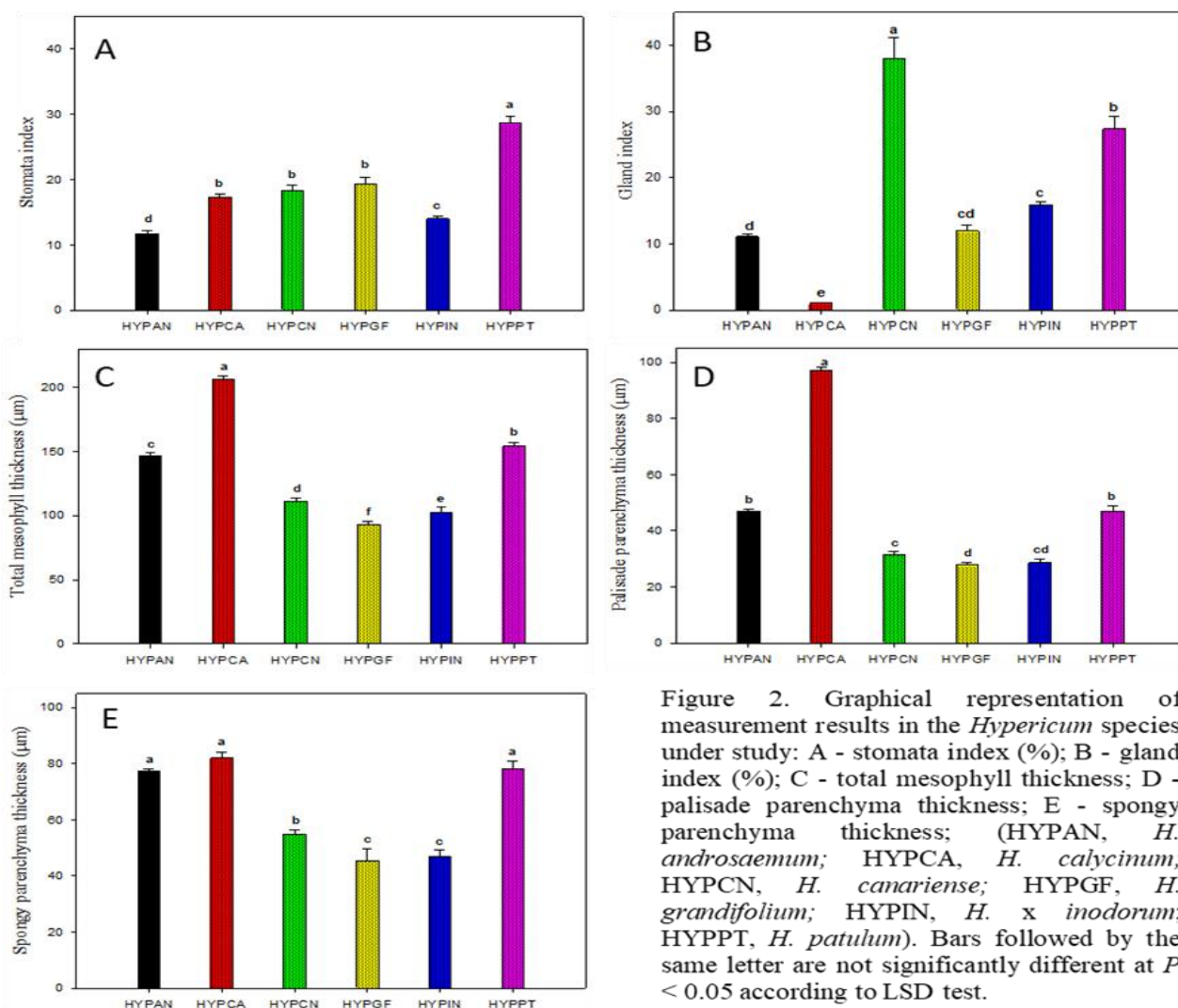


Figure 2. Graphical representation of measurement results in the *Hypericum* species under study: A - stomata index (%); B - gland index (%); C - total mesophyll thickness; D - palisade parenchyma thickness; E - spongy parenchyma thickness; (HYPAN, *H. androsaemum*; HYPCA, *H. calycinum*; HYPCN, *H. canariense*; HYPGF, *H. grandifolium*; HYPIN, *H. x inodorum*; HYPPT, *H. patulum*). Bars followed by the same letter are not significantly different at  $P < 0.05$  according to LSD test.

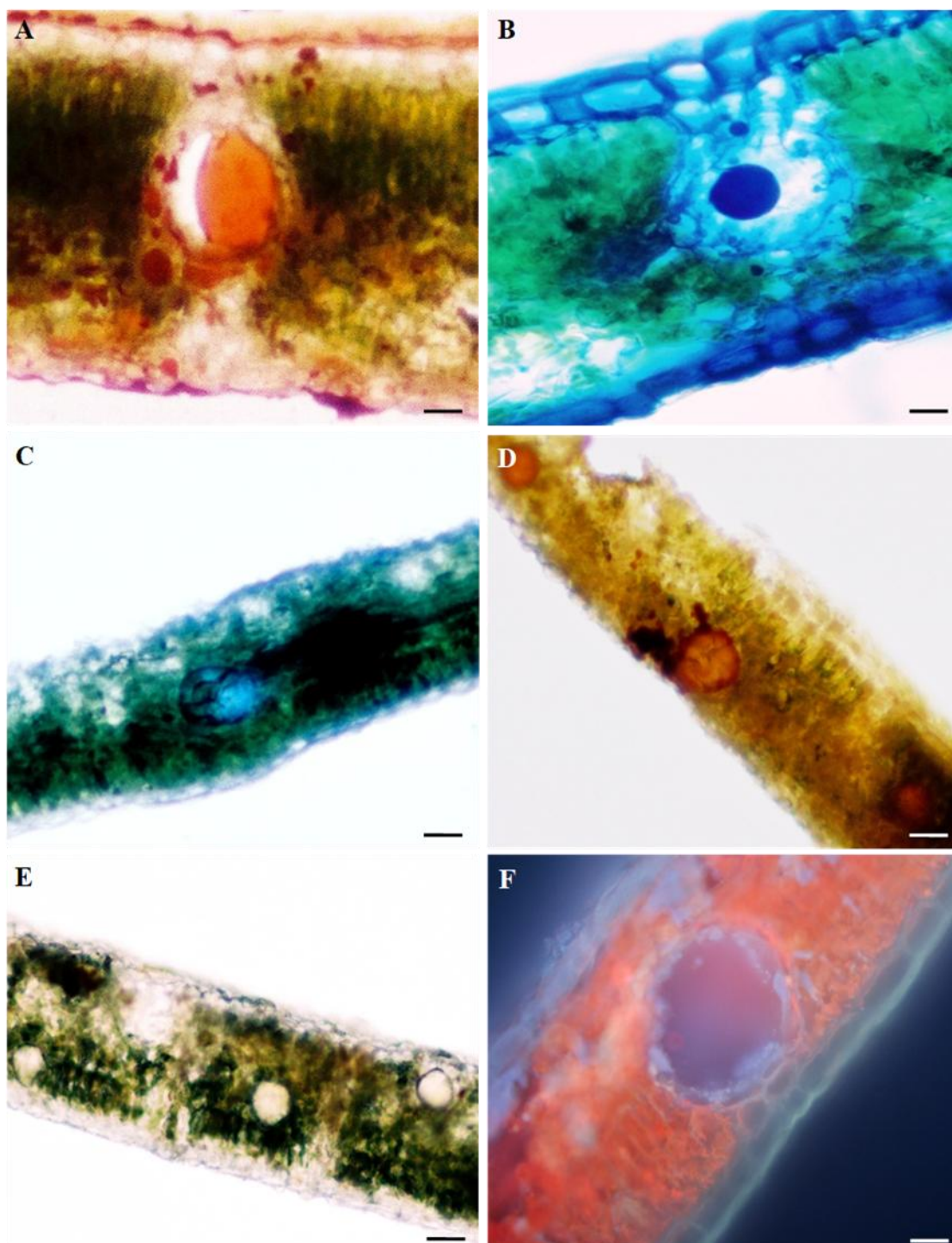


Figure 3. LM micrographs of leaves cross sections with internal secretory structures: A - *H. calycifolium* Sudan red positive for total lipids; B - *H. calycifolium* Nile blue positive for acid lipids; C - *H. patulum* Nadi positive for essential oils; D - *H. x inodorum* Dittmar positive alkaloids on glands; E - *H. x inodorum* Ferric trichloride glands negative and parenchyma positive; F - *H. calycinum* under UV light, autofluorescence of phenolic compounds in the cells surrounding the gland. (A, B and F scale bar = 12µm; C, D and E scale bar = 25µm).





Other secondary metabolites, such as the naphthodianthones hypericin and pseudohypericin are characteristic of the black nodules [7; 6] which were not present in the leaves of the studied species. This is very important when considering the use of *Hypericum* species for medicinal purposes, because the different types of secretory structures store distinct types of compounds [40; 45].

All taxa showed clearly bifacial leaves, confirming Metcalf and Chalk [40] data for *Hypericum* sp. The anatomical characterization provide some differentiation, at the mesophyll level when we consider its total thickness (Fig. 2C) we find three groups: i) *H. canariense*, *H. grandifolium* and *H. x inodorum* with values below 112  $\mu\text{m}$ ; ii) *H. androsaemum* and *H. patulum* with intermediate values up to 154  $\mu\text{m}$  and iii) *H. calycinum* with significantly higher values 206.4  $\mu\text{m}$ . Metcalf and Chalk [40] also mentioned that the mesophyll usually includes a single layer, occasionally two layers, of palisade tissue. In the six species under study only *H. calycinum* showed two layers of palisade parenchyma, with a thickness significantly higher than the other species (Fig. 2D). The species grouping as to the thickness of palisade parenchyma is similar to that of the previous set (Fig. 2D). When taking into account the average values of the spongy parenchyma thickness, only two groups significantly different are highlighted (Fig. 2E): i) *H. canariense*, *H. grandifolium* and *H. x inodorum* with values below 60  $\mu\text{m}$ , and ii) *H. androsaemum*, *H. patulum* and *H. calycinum* with values up to 90  $\mu\text{m}$ .

All taxa under study show standard dicotyledonous collateral vascular bundles arrangement, with a bundle sheath surrounding small and larger veins. *H. calycinum* was once again the exception, with fibrous sclerenchyma cells, with very thick cell walls and strongly

restricted lumen around xylem vessels, which isolates this vascular tissue from the ground tissue. Metcalf and Chalk [40] identified solitary and clustered crystals of calcium oxalate between the mesophyll cells in *Hypericum* species. We also found druses and raphids, but only in *H. calycinum* and *H. androsaemum*, respectively.

It is important to note that, in this study, the anatomical characters of the studied species do not fully confirm their taxonomic association in the current sections (Table 1) but further studies are needed.

#### IV. CONCLUSIONS

From the micromorphological analysis of the leaves of the studied species there are some differences between them which are new elements reported for the first time and which can be considered as phytognostic characters: the adaxial epidermal cell walls, the amount of epicuticular waxes, the stomata type, the stomata index, the glandular index and the mesophyll and each parenchyma thickness. Most of these characters are not influenced by environmental conditions, might be valid for the accurate and reproducible quality control of the commercial samples, allowing species differentiation and the detection of contaminants and adulterants. Considering our histochemical data, there are no significant differences between these species as they only indicate the presence / absence and the amount of some chemical groups and more data are needed.

#### ACKNOWLEDGEMENTS

We would like to thank Telmo Nunes, Faculdade de Ciências, Universidade de Lisboa, for his technical assistance with SEM; Teresa Vasconcelos, Instituto Superior de Agronomia,



Universidade de Lisboa for species identification and Silvia Costa for lab assistance.

### Funding

This study was financed by Portuguese National Funds, through FCT - Fundação para a Ciência e a Tecnologia, within the project UID/BIA/00329/2013.

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